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Evidence that PrfA, the Pleiotropic Activator of Virulence Genes in *Listeria monocytogenes*, Can Be Present but Inactive

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All virulence genes of *Listeria monocytogenes* identified to date are positively regulated by PrfA, a transcriptional activator belonging to the Crp-Fnr family. Low temperature and cellobiose are two environmental signals known to repress expression of virulence genes in *L. monocytogenes*. In the present work, we analyzed the effect of temperature and cellobiose on the expression of the PrfA protein. At low temperature, PrfA was undetected, although *prfA* monocistronic transcripts are present. In contrast, PrfA was fully expressed in the presence of cellobiose. These results strongly suggest that virulence gene activation depends on both the presence of PrfA and additional regulatory pathways that either modify PrfA or act synergistically with PrfA.

Listeria monocytogenes is a ubiquitous, gram-positive, facultative intracellular bacterium responsible for infrequent but often serious opportunistic infections in humans and animals. *L. monocytogenes* can grow in very different environments, from soil, plants, and water to intracellular mammalian tissues. It can support a wide range of temperatures (3 to 45°C), pHs (4.5 to 9), and osmolarity conditions (up to 10% NaCl). As a bacterial pathogen, it is capable of invading and multiplying in phagocytic and nonphagocytic cells (10, 16, 17). Its intracellular cycle is characterized by rapid phagolysosomal lysis followed by bacterial replication in the eucaryotic cytoplasm. Bacteria can spread within tissues by passing from an infected cell to adjacent cells by using an actin-based motility (2, 8). Eight genes involved in the infectious process have been identified. Six of these genes are clustered on the bacterial chromosome (Fig. 1A). *hly* encodes listeriolysin O, a pore-forming hemolysin involved in the lysis of the phagolysosomal membrane; *plcA* and *plcB* encode, respectively, a phosphatidylinositol-specific phospholipase C and a phosphatidylcholine-specific phospholipase C (also called lecithinase) involved in the lysis of bacterium-containing vacuoles; *mpl* encodes a metalloprotease necessary for the maturation of lecithinase; and *actA* encodes a surface protein absolutely necessary for actin polymerization (22). All of these genes are coordinately regulated by PrfA, the transcriptional activator encoded by the *prfA* gene (1a, 3, 13). On the basis of sequence similarities, PrfA was proposed to belong to the Crp-Fnr family of transcriptional activators. This conclusion has been strengthened recently by site-directed mutagenesis studies of the putative DNA binding domain of PrfA (20).

PrfA-mediated activation requires binding of PrfA at a conserved 14-bp dyad-symmetric site in PrfA target promoters (PrfA box) (4, 20). We have recently demonstrated a hierarchy in the *prfA*-regulated promoters (21). Activation by PrfA is more efficient at promoters which possess a perfectly symmetrical PrfA box than at promoters which have 1- or 2-bp substitutions in the PrfA box. *prfA* lies downstream from, and is cotranscribed with, *plcA*. It has been shown that *prfA* can be transcribed from three different promoters, i.e., the *plcA* promoter and the P1 and P2 *prfA*-specific promoters (Fig. 1B), producing a 2.1-kb bicistronic *plcA-prfA* transcript and 0.9- and 0.8-kb monocistronic *prfA* transcripts, respectively. Since PrfA activates transcription from the *plcA* promoter, it can positively regulate its own expression (13).

Environmental cues regulate expression of *L. monocytogenes* virulence genes as is often the case for bacterial pathogens (12). Temperatures below 37°C (9) and the presence of cellobiose in the bacterial medium (15) are two factors known to repress expression of *L. monocytogenes* virulence genes. Cellobiose is a disaccharide derived from the degradation of cellulose. It is probably present in large amounts in the soil where *L. monocytogenes* can grow saprophytically. Accordingly, it has been proposed that the bacterium, considered to be primarily a soil-borne organism, may use temperature and cellobiose concentration as environmental cues to sense its entry into a mammalian host and induce expression of genes required for infection. In the present work, we have examined the effect of both temperature and cellobiose on PrfA expression.

The amount of PrfA is reduced at low temperature. We and others have shown that transcription of virulence genes is repressed at low temperature (20 to 25°C) (3, 9). Although the level of *prfA* monocistronic transcripts was shown to be unaffected by temperature (9), the absolute quantity of *prfA*-specific transcripts was reduced at 20°C due to the absence of the *plcA-prfA* bicistronic message. By Northern (RNA) blot analysis, we confirmed and extended this result obtained for exponential-phase growing cultures to late exponential- and stationary-phase cultures (data not shown).

To compare PrfA levels at 20 and 37°C, we first raised antibodies against an N-terminal 18-amino-acid synthetic peptide of PrfA (20) and affinity purified them by using Sepharose affinity coupling gels (Pharmacia). The specificity of the affinity-purified antibodies was determined by Western blot (immunoblot) analysis of total protein extracts from wild-type *L. monocytogenes* ($PrfA^+$) compared to an isogenic PrfA deletion mutant (ΔPrfA) (21). A single band of approximately 30 kDa was observed in the wild-type strain. It was absent in the PrfA mutant. This band migrates at the expected position for PrfA (27 kDa) and represents the bona fide PrfA protein (Fig. 2A).

Protein extracts corresponding to the exponential, early stationary, and stationary phases of *L. monocytogenes* LO28 cultures grown in LB medium, at either 37 or 20°C, were then prepared by the method of Martin-Verstraete et al. (11) and analyzed by Western blotting. The results shown in Fig. 2B

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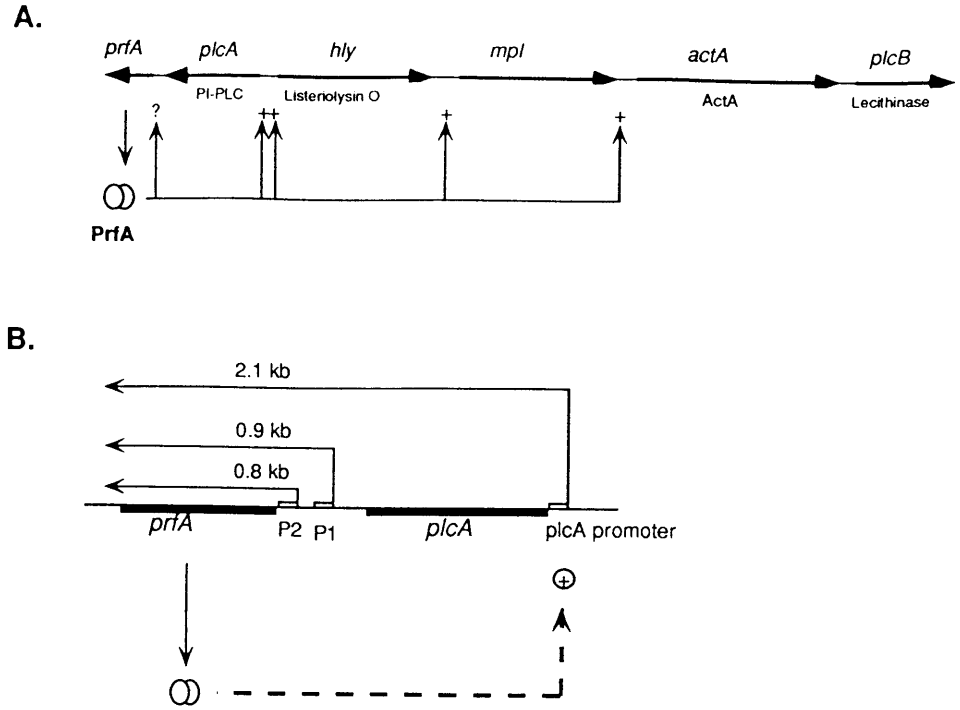

FIG. 1. Regulation of virulence genes by PrfA in *L. monocytogenes*. (A) Schematic representation of the virulence gene cluster activated by PrfA; (B) *prfA* gene transcription. Arrows indicate *prfA* transcripts produced from the *plcA* promoter and from the P1 and P2 *prfA* promoters. PI-PLC, phosphatidylinositol-specific phospholipase C.

indicate that PrfA is present at 37°C all along the growth curve while at 20°C the PrfA protein is undetectable although *prfA* monocistronic transcripts are present. It is important to note that under the same conditions, some listerial proteins such as flagellin are fully expressed (19). To test whether the *prfA* monocistronic transcripts can be translated, we examined the level of PrfA in CDI5, an *L. monocytogenes* strain with a transposon insertion in *plcA*, in which *prfA* is expressed only as monocistronic messages (5). Western blot analysis of protein extracts from the parental strain *L. monocytogenes* LO28 and the isogenic mutant CDI5 shows that both strains are able to produce detectable amounts of PrfA protein at 37°C (Fig. 2C). In CDI5, the amount of PrfA was nevertheless slightly reduced compared to that of the parental strain, probably due to the absence of the *prfA* bicistronic message (Fig. 2C). These observations suggest that the undetectable levels of PrfA at low temperature are not due to the untranslatability of monocistronic PrfA messages.

The amount of PrfA protein is not affected by the presence of cellobiose. Cellobiose, like temperature, is known to downregulate transcription of virulence genes. In bacterial cultures containing cellobiose (1 to 100 mM), transcription of *hly* and *plcA* is repressed (15). However, as previously noticed, this repression does not correlate with a decrease in *prfA* transcript levels (5). We thus examined in detail the effect of cellobiose on *prfA* transcription. Northern blot analysis of total RNA from LO28 exponential- and stationary-phase cultures grown in the presence or absence of cellobiose showed that in the exponential phase, the three types of *prfA* transcripts were present (the 2.1-kb bicistronic transcript and the 0.9- and 0.8-kb monocistronic transcripts), but in the presence of cellobiose there was a decrease in the quantity of bicistronic transcript and a shift from the P2 promoter (0.8-kb monocistronic transcript) to the P1 promoter (0.9-kb monocistronic transcript). We also noticed that in the exponential phase, in either the presence or absence of cellobiose, the total amounts of *prfA* transcripts were roughly similar (Fig. 3A, left panel). In the stationary phase, the total amounts of *prfA* transcripts decreased both in the absence of cellobiose, as already described (13), and in its presence. Interestingly, there was a relatively higher amount of the small P1 transcript in the presence of cellobiose than in its absence.

Since the overall *prfA* transcription was not dramatically changed by the presence of cellobiose, we then tested whether the production of PrfA was reduced by the presence of cellobiose. Total protein extracts of bacteria grown in LB medium with or without cellobiose (50 mM), corresponding to the exponential, early stationary, and stationary phases, were analyzed by Western blotting with the PrfA-specific antibodies. As shown in Fig. 3B, PrfA was detected at similar levels in cultures grown in the presence or absence of cellobiose. We even noticed a slight increase in the PrfA level in the stationary-phase culture with cellobiose (Fig. 3B) that correlates with the increase of *prfA*-specific transcripts observed in the stationary phase in the presence of cellobiose (Fig. 3A). Under the same culture conditions, the amounts of listeriolysin O (58 kDa) and ActA (90 kDa) were greatly reduced in the presence of cellobiose as shown by Western blot analysis (Fig. 3B). Taken together, our results demonstrate that cellobiose reduces the levels of virulence factors while there is no reduction in the levels of the activator protein PrfA.

To test whether cellobiose directly affects binding of PrfA to its target promoters, we made use of a transcriptional *hly-lacZ* fusion previously constructed in *Bacillus subtilis* (21), since it is known that cellobiose is taken up by *B. subtilis* via a cellobiose-specific phosphotransferase system (7). The ability of PrfA to activate the expression of the *hly-lacZ* fusion in the presence or absence of cellobiose was tested by measuring the β-galacto-

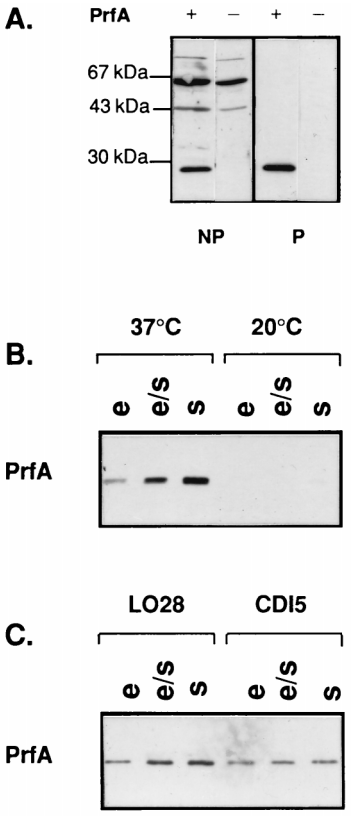

FIG. 2. Detection of PrfA protein. (A) Affinity purification of anti-PrfA antibodies. Six micrograms of total protein extracts of wild-type LO28 (+) and of PrfA mutant (−) were run in a 10% acrylamide gel and analyzed by Western blotting with an unpurified (NP) and purified (P) anti-PrfA antiserum (dilution, 1/2,000). The antiserum was previously raised against an 18-amino-acid synthetic peptide corresponding to residues 15 to 31 of PrfA (20). Purification was performed with Sepharose affinity coupling gels (Pharmacia). Briefly, the synthetic peptide was coupled to ECH Sepharose 4B by use of the carbodiimide EDC. Coupling was done at pH 4.5 and 4°C overnight. After blocking the uncoupled sites, antiserum was applied. Antipeptide antibodies were eluted with 100 mM glycine (pH 2.5) and stored at −70°C in 50% glycerol–PBS. (B) Effect of temperature on PrfA levels. *L. monocytogenes* LO28 was cultured in LB medium at either 37 or 20°C and harvested during the exponential (e), early stationary (e/s), and stationary (s) phases. Approximately 6 μg of total protein extracts was loaded per well in a 10% acrylamide gel. PrfA protein was detected by Western blotting with the specific anti-PrfA antibodies. (C) Expression of PrfA in the mutant CDI5. The analysis was the same as that described for panel B but with wild-type *L. monocytogenes* LO28 and the isogenic mutant CDI5.

sidase activity as described previously (5). We found only a slight reduction in the β-galactosidase activity in cultures grown in the presence of cellobiose compared to that in cultures grown in the absence of cellobiose (2,292 ± 65 fluorescence units versus 2,734 ± 64 fluorescence units [mean ± standard deviation]). These results are different from the situation in *L. monocytogenes*, where *hly* expression is reduced by at least 20-fold in the presence of 50 mM cellobiose (15). These earlier results strongly suggest that cellobiose does not act directly on PrfA protein. However, one should keep in mind that in *B. subtilis*, cellobiose is converted to cellobiose-PO_4 during its transport. It may not be the case in *L. monocytogenes*. It thus remains possible that cellobiose can act as a PrfA corepressor in *L. monocytogenes*. This question deserves further investigation.

In summary, we present evidence for two distinct mechanisms involved in the repression of virulence gene expression by two environmental cues, low temperature and presence of cellobiose. These two factors have different effects on the expression of PrfA, the pleiotropic transcriptional activator of virulence genes. Whereas temperature modulates the absolute amounts of the activator protein PrfA, cellobiose does not affect the amount of PrfA and probably acts posttranslationally.

At low temperature, there is a reduction in the total levels of

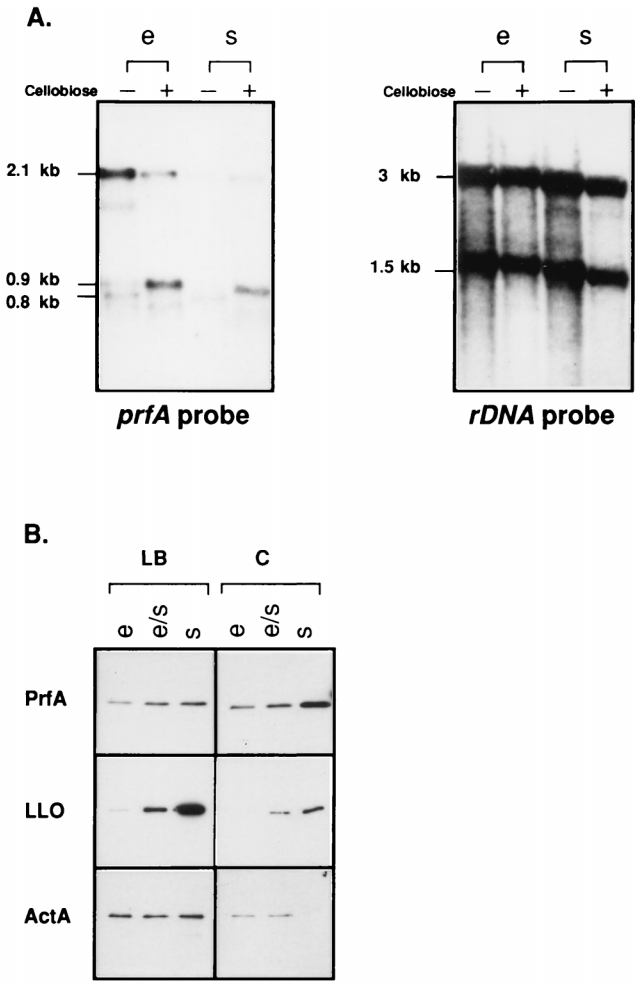

FIG. 3. Effect of cellobiose. (A) Effect of cellobiose on *prfA* transcription. Total RNA was prepared from wild-type LO28, grown in LB medium with (+) or without (−) cellobiose, and harvested during exponential (e) and stationary (s) phases as described previously by Sheehan et al. (21). Northern blot hybridization was performed as described by Sheehan et al. (21) with, as the *prfA*-specific probe, a PCR DNA fragment spanning positions 321 to 690 in the DNA sequence of *prfA* gene (13). mRNA sizes were calculated by use of the RNA markers from Novagen. For the left panel, 6 μg of total RNA was loaded per well, and *prfA* gene transcripts were detected with a *prfA*-specific probe; for the right panel, the Northern blot shown in the left panel was hybridized with a ribosomal DNA probe. (B) Effect of cellobiose on the expression of PrfA, listeriolysin O (LLO), and ActA proteins. *L. monocytogenes* LO28 was cultured at 37°C, in LB medium (LB) or LB medium containing 50 mM cellobiose (C), and harvested at the exponential (e), early stationary (e/s) and stationary (s) phases. For PrfA protein, detection of PrfA was performed by Western blotting as described in the legend to Fig. 2B. For listeriolysin O protein, we have analyzed the expression of listeriolysin O in culture supernatants since LLO is a secreted protein. Proteins from culture supernatants were precipitated by adding 2 volumes of acetone and incubating on ice for 40 min. Precipitated proteins were collected by centrifugation at 4,000 × *g* for 20 min and analyzed with a specific anti-listeriolysin O monoclonal antibody (D21-1-4) (14). Loading corresponds to 100 μl of culture supernatants. For the ActA protein, identical amounts of total protein extracts were loaded in each lane and analyzed for the presence of ActA by using affinity-purified anti-ActA antibodies (Y21T) (6).

prfA transcripts and a reduction in the amounts of PrfA. Thus, temperature modulates the levels of PrfA to reduce virulence gene expression. Nevertheless, we cannot exclude the possibility that other factors or conformational changes in PrfA play a role in the reduction of virulence gene transcription at low temperature.

In the presence of cellobiose, the reduced expression of virulence genes is not due to a decrease in the amount of PrfA produced. Indeed, the quantity of PrfA does not change in the presence of cellobiose. These data are in agreement with the fact that the total amount of *prfA* transcripts is not affected by the presence of cellobiose, even though the presence of cellobiose changes the relative amounts of the three PrfA-encoding transcripts. Taken together, these data support our previous hypothesis (5) that cellobiose could reduce the levels of active PrfA rather than PrfA synthesis. Cellobiose could have directly affected the mechanism of *prfA* gene activation, as was found for several transcriptional repressor proteins which bind carbohydrates for their function (18). Our results showing that in *B. subtilis* the ability of PrfA to activate the *hly* promoter was not repressed by the presence of cellobiose suggest but do not establish that cellobiose does not interact directly with PrfA and that there are other listerial factors involved in the cellobiose regulatory pathway. We are currently investigating this possibility. Our results are in complete agreement with a recent paper which demonstrates that PrfA-mediated activation requires the presence of a coactivator protein (1).

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